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Condensed tannins as antioxidants that protect poplar against oxidative stress from drought and UV-B

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Abstract

Condensed tannins (CTs, proanthocyanidins) are widespread polymeric flavan-3-ols known for their ability to bind proteins. In poplar (*Populus* spp.), leaf condensed tannins are induced by both biotic and abiotic stresses, suggesting diverse biological functions. Here we demonstrate the ability of CTs to function as physiological antioxidants, preventing oxidative and cellular damage in response to drought and UV-B irradiation. Chlorophyll fluorescence was used to monitor photosystem II performance, and both hydrogen peroxide and malondialdehyde content was assayed as a measure of oxidative damage. Transgenic MYB-overexpressing poplar (*Populus tremula* × *P. tremuloides*) with high CT content showed reduced photosystem damage and lower hydrogen peroxide and malondialdehyde content after drought and UV-B stress. This antioxidant effect of CT was observed using two different poplar MYB CT regulators, in multiple independent lines and different genetic backgrounds. Additionally, low-CT MYB134-RNAi transgenic poplars showed enhanced susceptibility to drought-induced oxidative stress. UV-B radiation had different impacts than drought on chlorophyll fluorescence, but all high-CT poplar lines displayed reduced sensitivity to both stresses. Our data indicate that CTs are significant defences against oxidative stress. The broad distribution of CTs in forest systems that are exposed to diverse abiotic stresses suggests that these compounds have wider functional roles than previously realized.

KEYWORDS
abiotic stress, flavan-3-ols, flavonoids, oxidative stress, plant physiology, *Populus*, proanthocyanidins, reactive oxygen species

1 | INTRODUCTION

Condensed tannins (CTs), also known as proanthocyanidins, are widespread plant secondary metabolites with diverse ecological functions. They are major end products of the flavonoid pathway and consist of polymeric flavan-3-ols ranging in size from 2 to 30 or more subunits (Dixon et al., 2005; Quideau et al., 2011). The CTs are particularly common in woody species, where they accumulate in vegetative tissues, including leaves, roots and bark (Barbehenn & Constabel, 2011). They are present in both conifers and broad-leaved trees and shrubs, including many keystone species in temperate forests (Mole, 1993). In herbaceous plants, CTs are generally restricted to the seed coat but are occasionally found in flowers and leaves. CTs can be found in many food plants, in particular in grains, nuts and berry fruit, as well as in plant-based beverages, such as beer and red wine. Diets rich in CTs are considered beneficial, as high CT intake is correlated with a reduced risk of cardiovascular disease, neurodegenerative conditions and other chronic diseases (Prior & Gu, 2005). CTs are excellent in vitro antioxidants, mainly due to the propensity of phenolics for the donation of a hydrogen atom and for single-electron transfer (Quideau et al., 2011).

The antioxidant activity of CTs is enhanced by their high molecular weight and proximity of aromatic rings and hydroxyl groups (Hagerman et al., 1998).

CTs are linked to an astonishing diversity of ecological functions. Like other tannins, CTs can bind and precipitate proteins. As a result, CTs were originally proposed to be antinutritive defenses against leaf-eating insects. However, CTs generally show low efficacy against lepidopterans, though at moderate to high concentrations they can be effective defenses against other insects and vertebrates (Barbehenn & Constabel, 2011). CTs also have broad antimicrobial activity (Scalbert, 1991), with effects in plants and the environment. For example, CTs contribute to defense against *Melampsora*, a biotrophic pathogen of poplar (Ullah et al., 2017). In forest soils, litter-derived CTs inhibit soil microbial activity, slowing litter decomposition and nutrient cycling (Hättenschwiler & Vitousek, 2000). CTs in forages such as sainfoin (*Onobrychis viciifolia*) regulate rumen microbial activity in cattle and other ruminants, reducing the risk of foaming and bloat (Waghorn & McNabb, 2003). Thus, CTs have both ecological and practical importance.

Condensed tannin biosynthesis is generally well understood, in part based on extensive studies in Arabidopsis (W. J. Xu et al., 2015). Both CT-specific and general flavonoid enzymes have been identified and characterized. However, important questions remain regarding the mechanism of polymerization, transport to the vacuole and other potential sites of localization (Dixon et al., 2005). The pathway is transcriptionally regulated by the MYB-bHLH-WDR (MBW) transcriptional complex. In Arabidopsis, the TT2 MYB transcription factor is the major determinant of expression (W. J. Xu et al., 2015). Additional TT2-like MYB regulators of the CT pathway have been characterized in other model systems, including *Medicago trunculata*, grapevine (*Vitis vinifera*) and poplar (*Populus*). For functional studies, poplar is an excellent model, with extensive genome databases, efficient transformation protocols and many ecological interactions (Jansson & Douglas, 2007). Poplar leaves, bark and roots all produce large amounts of CT, with some species accumulating up to 25% of leaf dry weight as CTs (Lindroth & Hwang, 1996). The key regulatory genes of CT synthesis in poplar have been identified, including both MYB activators and repressors (James et al., 2017; Ma et al., 2018; Mellway et al., 2009). MYB134 and MYB115 transcription factors were characterized as specific regulators of the CT pathway in poplar and shown to interact with flavonoid and condensed tannin gene promoters in MBW complexes in vivo. Importantly, when overexpressed in transgenic poplar plants under the control of a strong constitutive promoter, both MYB genes caused a 50-fold increase in CTs, but only very minor changes in other flavonoids (Mellway et al., 2009). Poplar CTs are composed primarily of procyanidin subunits, with varying proportions of prodelphidin subunits, which vary by tissue type and are also enhanced by MYB overexpression (James et al., 2017). The specificity of MYB115 and MYB134 for the CT pathway was further confirmed using transcriptomic analysis. The high-CT transgenic poplars are thus extensively characterized and have become powerful tools for testing ecological functions of the CTs (Boeckler et al., 2014; Gourlay & Constabel, 2019; Ullah et al., 2017).

A key feature of CT biosynthesis in *Populus* is its regulation by both developmental and environmental signals. In particular, different stresses, such as herbivory, pathogen infection, UV-B radiation, high light exposure and nitrogen deficiency can stimulate CT biosynthesis (Gourlay & Constabel, 2019; Harding et al., 2005; Mellway et al., 2009). These abiotic and biotic stresses differ in the types of cellular damage or metabolic disruptions they cause. However, all stimulate the accumulation of reactive oxygen species (ROS) and lead to oxidative stress. Since CTs are potent antioxidants in vitro (Hagerman et al., 1998; Quideau et al., 2011), CT pathway induction by diverse stresses thus suggests that CTs may function as physiological antioxidants as well. The high CT content in leaves of forest trees makes it an attractive hypothesis. Furthermore, other flavonoids, such as anthocyanins, have been found to function as antioxidants in vivo (Nakabayashi et al., 2014; Z. Xu et al., 2017). We previously demonstrated that in detached leaves high CT levels protect tissues against methyl viologen, a superoxide-generating herbicide (Gourlay & Constabel, 2019; Gourlay et al., 2020). However, it is not known if CTs are effective antioxidants in planta and if they can contribute to the protection of intact poplar plants against abiotic stresses such as drought or UV-B exposure.

Abiotic stresses have complex and diverse effects on plant cells, including metabolic disturbances, inhibition of growth and cell death and ROS generation (Apel & Hirt, 2004; Gill & Tuteja, 2010). While ROS are typically generated as normal by-products of metabolism, during adverse conditions they accumulate to higher levels and exceed rates of removal, thus causing oxidative stress (Apel & Hirt, 2004; Czarnocka & Karpiński, 2018). In photosynthetic leaves, a major source of ROS during abiotic stress is disruption of chloroplasts and leakage of electrons from photosynthetic electron transport chains (Demidchik, 2015; Gill & Tuteja, 2010). For example, during drought, the stomata close to prevent water loss, which reduces the leaf capacity for CO_2 assimilation and leads to an overreduction of the electron transport chain in the chloroplast, generating superoxide via the Mehler reaction (Cruz de Carvalho, 2008; Miller et al., 2010). Superoxide is short-lived but can lead to the generation of highly reactive hydroxyl radicals (Choudhury et al., 2017; Demidchik, 2015). UV-B exposure, by contrast, can directly damage photosystem components and Calvin cycle enzymes. Electron transport malfunction then leads to the formation of superoxide and other ROS, which can then damage other cellular components (Foyer et al., 1994; Kataria et al., 2014). Excess UV-B also causes the direct production of hydroxyl radicals in thylakoid membranes, leading to disruption of photosynthesis and additional ROS (Czégény, Mátai et al., 2016).

Since ROS generated by both drought and UV-B stress ultimately damage the photosynthetic apparatus, measuring photosystem function by chlorophyll fluorescence is commonly used as nondestructive measure of the impact of abiotic stress (Murchie & Lawson, 2013). It provides a direct assessment of photosystem functionality, or conversely, photosystem damage (Maxwell & Johnson, 2000). An indirect measure of damage by ROS is provided by quantifying lipid peroxidation products. Lipid peroxides are formed by the interaction of ROS with membranes, causing propagating chain reactions, further membrane damage and ultimately a break-down of organelles (Demidchik, 2015; Farmer & Mueller, 2013). These reactions generate additional lipid radicals and end products, most commonly malondialdehyde (MDA). MDA is a convenient biochemical marker for oxidative stress since most ROS are very short-lived and

difficult to measure (Noctor et al., 2015). One exception is H_2O_2, which is longer-lived and can be quantified in fresh plant extracts. Therefore, H_2O_2 and MDA levels in plant tissues, together with chlorophyll fluorescence, are commonly used indicators for oxidative stress in plants.

To respond to oxidative stress and detoxify ROS, plants use a battery of enzymatic and nonenzymatic mechanisms. Superoxide is rapidly detoxified by superoxide dismutase (SOD), a ubiquitous enzyme that converts superoxide to H_2O_2. SODs can be found in several cellular compartments, including peroxisomes, mitochondria and plastids. H_2O_2 is then converted to water by catalases. In addition, ascorbate peroxidase (APX EC 1.11.1.11) and glutathione peroxidase (GRx EC 1.11.1.9) convert H_2O_2 to water as part of the Foyer-Asada-Halliwell (glutathione-ascorbate) cycle (Noctor & Foyer, 1998; Pandey et al., 2017). Ascorbate and glutathione are both abundant soluble antioxidants that scavenge ROS directly (Apel & Hirt, 2004; Noctor et al., 2014). Plant cells also use α-tocopherol (vitamin E) as a lipid-soluble antioxidant (del Río, 2015). Additional nonenzymatic mechanisms for scavenging ROS produced during photosynthesis also include the xanthophyll cycle and the water–water cycle (Asada, 1999; Demmig-Adams, 1990). In poplar and some other tree species, isoprene is emitted from leaves during heat, drought and other abiotic stresses. Its function in plant stress tolerance is not completely understood, but it has been proposed to function as an antioxidant or stabilize photosynthetic membranes following stress (Behnke et al., 2010; Ryan et al., 2014).

Here, we demonstrate that foliar CTs function as additional nonenzymatic antioxidants that can protect leaves of poplar saplings against the oxidative damage caused by drought and UV-B. We use our extensively characterized MYB134- and MYB115-overexpressor transgenics to show that high-CT plants sustain substantially less photosystem II (PSII) damage and accumulate less H_2O_2 and MDA than similarly stressed control plants. Conversely, MYB134-RNAi poplars with reduced CT content manifest greater oxidative damage. The protective role of these widespread compounds against both drought and UV-B radiation, two distinct ROS-producing abiotic stresses, supports our hypothesis that CTs can act as general antioxidants in trees.

2 | MATERIALS AND METHODS

2.1 | Plant growth conditions and treatment

Populus tremula × *Populus tremuloides* L. (clone INRA 353-38) wild-type (WT), high-CT transgenic MYB134- and MYB115-overexpressing lines (James et al., 2017; Mellway, Tran, et al., 2009) and low CT MYB134-RNAi plants (Gourlay et al., 2020) were available in the Constabel laboratory and propagated as described. For experiments, plantlets were acclimated and grown for 2 months in a glasshouse (Major & Constabel, 2006) with supplemental lights to extend day length (average intensity at 300 µmol photons $m^{-2}\,s^{-1}$). Drought experiments were carried out in the Glover Greenhouse at the University of Victoria (latitude 48.46°N) with four individual replicates per independent poplar line per treatment. Before experiments, pots were first saturated with water. Drought-treated plants received 20–30 ml of water 3× per day, while well-watered plants received 100–150 ml of water 3× daily, depending on plant size. When pot weight stabilized in the drought-stressed plants (<10 g difference between days), the plants were considered to be in drought (Sinclair & Ludlow, 1986). To start recovery, pots were saturated with water.

For MYB134-RNAi experiments, both RNAi and WT plants were first induced by spraying with methyl jasmonate (MeJa) on Days 0, 2, 4 and 6, as described previously (Gourlay et al., 2020), and drought was initiated on Day 10. This pretreatment was required to stimulate CT biosynthesis in young poplar and led to a stronger differentiation between MYB134-RNAi and WT plants (Gourlay et al., 2020).

For UV-B experiments, nodal tissue culture cuttings were shipped to Helmholtz Zentrum München. In vitro grown plants were acclimated and grown for 8 weeks in a glasshouse and then moved into 'sun simulator' chambers. One chamber was programmed to simulate natural sunlight exposure similar to latitudes in southern Europe (latitude 40°N), including UV-B (290–315 nm) and UV-A (315–400 nm) radiation (Thiel et al., 1996). In parallel, an identical control chamber exposed plants to simulated sunlight but without UV-B. Before UV-B treatments, all plants were acclimated to the chambers for 1 week, during which photosynthetically active radiation (PAR) was increased gradually from 500 to 1400 µmol photons $m^{-2}\,s^{-1}$. During the UV-B treatment phase, plants were exposed to visible wavelengths for 16 h (PAR intensity: 1400 photons $m^{-2}\,s^{-1}$) and UV-B for 13 h (daily biologically effective UV-B: 46.8 kJ m^{-2}) (Kaling et al., 2015). Temperatures were set to 27°C during the day and 17°C at night (relative humidity 40% and 80%, respectively). Plants were kept in trays filled with a small amount of water and were rotated daily. There were six individual replicates per poplar line per treatment.

Chlorophyll fluorescence measurements for drought experiments were conducted with an OPTI-Sciences Modulated Chlorophyll Fluorometer OS1p (Opti-Sciences, Inc.) as outlined (Gourlay & Constabel, 2019). All fluorescence measurements were taken at leaf 10. Due to the sensitivity of F_q'/F_m' (PS II operating efficiency) to light flecks, fluorescence measurements during the drought experiment were taken predawn under artificial glasshouse lights (300 µmol photons $m^{-2}\,s^{-1}$ at the leaf being measured; PL2000 MIDI 600 W, P. L. Light Systems). Artificial lights had been on for at least 60 min to ensure full activity of the photosystems.

For the UV-B experiments, chlorophyll fluorescence was measured with a miniPAM fluorometer (Walz). Both F_q'/F_m' and F_v/F_m were recorded early each day (measurements were taken at approximately 7:30 AM). Dark acclimation clips for F_v/F_m measurements were placed in three similar locations on each leaf and the chamber was darkened for 30 min before the measurement. All measurements were taken at the same time of day for each replicated experiment.

2.2 | Morphological and physiological measurements

Stem diameter 10-cm from the soil and plant height were taken at key time points (time zero, beginning of the drought, end of the drought, end

of recovery) during drought experiments. For UV-B experiments, measurements were taken at Day 0, Week 1 of UV-B exposure and Week 2 of UV-B exposure. Gas exchange analysis during drought experiments was performed weekly using an LCA4 portable gas exchange system (ADC Bioscientific Ltd., Global House) on leaf 10. Measurements were taken at mid-day for optimal and consistent light intensity. Water use efficiency (WUE) was recorded as μmol CO_2 per mmol H_2O. For drought experiments, the number of necrotic leaves (leaves that showed clear browning at the leaf edges or general necrosis) was recorded daily. For UV-B experiments, gas exchange measurements were taken as described by Kaling et al. (2015) on leaf 10 using a portable gas exchange system, GFS-3000 (Walz). For UV-B experiments, relative indices of flavonol and chlorophyll measurements were estimated with a Dualex Multiplex 3.6 handheld optical sensor (Force A, Centre entrepreneurial de l'Institut d'Optique, Orsay, France) every 3–4 days at the same time of day. Three measurements per leaf were taken on the adaxial side of each leaf for mature leaves (leaves 10–11) and young leaves (leaves 2–3).

2.3 | RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from poplar leaves as previously described (Yoshida et al., 2015), treated with RQ1 DNase (Promega) and used to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. RT-qPCR was performed in 20 μl reactions using 4 μl homemade qPCR master mix on a CFX96 Real Time system (Bio-Rad). The master mix (final concentrations) contained 24.1 mM Tris-HCl, 25.9 mM Tris-base, 25 mM KCl, 1.5 mM $MgCl_2$, 0.5% (v/v) Tween 20, 4% (v/v) glycerol, 0.83 mM dNTP and 5X EVAGreen SYBR (Biotium) in a total volume of 2 ml. Transcript abundance for MYB134 (Potri.006G221800) was normalized using the geometric mean of poplar elongation factor (EF1β; Potri.001G224700) and ubiquitin (UBQ10; Potri.014G115100) expression (Yoshida et al., 2015).

2.4 | Phytochemical and ROS analysis

Twenty-five milligrams of ground, freeze-dried tissue was weighed into 2-ml CryoTubes with four steel beads. Tissue was homogenized and extracted in 1.5 ml of 100% MeOH using a Precellys tissue homogenizer (Bertin Technologies, Rockville, MD, USA) for 2 × 45 s at 5000 rpm followed by centrifugation of 10 min at 15 000*g*. Extractions were repeated twice more with an additional 1 ml MeOH each, giving 3.5 ml of total plant extract. CT analysis was carried out using the butanol-HCl method as outlined (Gourlay & Constabel, 2019). Anthocyanins were extracted and assayed spectrophotometrically following Ma et al. (2018).

Isoprene emission rates were assayed by proton transfer reaction mass spectrometry (PTR-MS) according to Behnke et al. (2010). Three 1-cm diameter leaf discs were excised from leaf 10 at noon and placed in a glass vial with 1 ml of CO_2− infused water. The vial was left open for 30 min while acclimation occurred and then was sealed, and the vials were placed on a lightbox for 2 h after which the amount of isoprene emitted was measured. There were six biological replicates per line for each treatment. Emission rates were normalized to standard conditions (1000 μmol m^{-2} s^{-1} and 30°C) (Behnke et al., 2010).

Hydrogen peroxide levels were assayed on harvested leaves as outlined in Gourlay and Constabel (2019). MDA concentrations were determined based on Yu et al. (2015) and Yang et al. (2015) with modifications as follows. Fifty milligrams of frozen leaf powder were weighed into a cooled 2-ml CryoTube with four steel beads, 1.5 ml of 10% (v/v) trichloroacetic acid (TCA) was added, and samples homogenized using a Precellys tissue homogenizer for 2 × 45 s at 5000 rpm. Following centrifugation (10 min at 12 000*g* at 4°C), 1 ml of 0.6% (v/v) thiobarbituric acid (TBA) in 10% (v/v) TCA was added to 1 ml of supernatant and the assay mixture incubated at 95°C for 30 min in a water bath. Samples were cooled in an ice bath, centrifuged for 10 min at 4°C and absorbance read at A_{400}, A_{532}, A_{600} on a spectrophotometer (Thermo Spectronic Genesys 10uv Scanning spectrophotometer, ThermoFisher Scientific). Total MDA content was calculated according to Yang et al. (2015). Since high CT content in extracts generated high background absorbance readings in the MDA assay, we carried out control assays containing assay reagents minus TBA (same volume; TBA was substituted with 1 ml of TCA). These were used for background corrections.

2.5 | Statistical analyses

Data were analysed using *t* tests of means, analysis of variance (ANOVA) or repeated-measures ANOVA and Tukey honest significant difference (HSD) post-hoc tests in R (https://www.r-project.org); details are presented in the figure legends. Sources of variation for ANOVAs were genotype, treatment and their interaction, or genotype, treatment, time and their interactions.

3 | RESULTS

3.1 | High CT content protects poplar leaves against drought-induced damage to PSII and reduces ROS accumulation

To test the effect of high CT content on drought tolerance of poplar, we induced drought stress on two high-CT MYB-overexpressing and control *P. tremula* × *P. tremuloides* poplars by reducing water supply. We used two independently transformed transgenic MYB134- and MYB115-overexpressor lines (James et al., 2017; Mellway, Tran, et al., 2009). Under the drought conditions imposed, pot weight stabilized between Day 8 and Day 10; we defined the onset of drought based on this (Figure 1, Figure S1). Stomatal conductance measurements confirmed that the plants were in drought after 5 days of reduced watering (Figure S2). The drought treatment clearly affected all plant lines equally, observed as net CO_2 assimilation, reduced stomatal conductance and transpiration (Figure S3). All

drought-treated plants showed drastically reduced growth rates and were significantly smaller than well-watered plants at the end of the experiment (Figure 1). Both the MYB-overexpressing and control plants showed a similar reduction in gas exchange and growth.

The progressive effect of drought stress on the plants was monitored by chlorophyll fluorescence. F_q'/F_m' remained constant in well-watered control plants throughout drought experiments (Figure 2a,b). Beginning on Day 11 of drought, WT plants showed a strong reduction in F_q'/F_m' in leaves that continued until the recovery period. In contrast, both MYB134OE and MYB115OE transgenics demonstrated only a moderate decline in fluorescence (Figure 2a,b), indicating that PSII in high-CT plants was less affected by drought stress than in WT plants. This suggested that high concentrations of CTs could protect against the effects of drought. After rewatering, fluorescence increased gradually as the plants began to recover. However, within the time frame of our experiments, they did not return to initial starting values. To confirm these observations, we repeated the experiment with MYB134-overexpressor transgenics created in a different poplar hybrid, *P. tremula* × *Populus alba* (clone 717-B4) (Mellway, Tran, et al., 2009). These plants have the same high-CT phenotype as the *P. tremula* × *P. tremuloides* plants used above. Again, a strong protective effect of the CTs against the negative effects of drought on PSII was observed: although drought affected all the plants, F_q'/F_m' chlorophyll fluorescence remained higher in MYB134-overexpressing plants compared to control plants (Figure S4).

We next investigated if the reduced PSII damage in MYB134- and MYB115-overexpressors after drought stress was due to lower concentrations of ROS. At the end of the drought experiment, we quantified H_2O_2 in leaf extracts. Only non-necrotic leaves were sampled. In high-CT lines, significantly lower H_2O_2 content was observed compared to WT leaves (Figure 2c). Drought induced an increase in H_2O_2 content in WT plants as well as MYB overexpressors, but overall levels were lower in high-CT lines under both drought and well-watered conditions compared to controls. Likewise, after drought, the high-CT transgenics had almost three-fold lower MDA content compared to WT (Figure 2d). We also observed a strong correlation between H_2O_2 and MDA concentrations (Figure 2d, inset). These data suggest that reduced PSII damage in high-CT plants after the drought was likely due to reduced ROS build-up and less oxidative damage.

3.2 | Reduced foliar CT content makes transgenic poplar more susceptible to oxidative damage caused by drought

Our results with high-CT plants suggested that poplar with low CT content should be more susceptible to the effects of oxidative stress. We, therefore, conducted additional drought experiments with MYB134-RNAi poplars, which have a reduced capacity to synthesize CTs (Gourlay et al., 2020). Because glasshouse-grown poplars (including WT) generally contain very little CT unless plants are stressed, we applied a MeJa pretreatment to induce CTs above baseline levels (Gourlay et al., 2020), after which drought stress was imposed. In these experiments, the onset of drought occurred on Day 6. The drought treatment was stopped after 10–12 days due to the greater drought sensitivity of MYB134-RNAi plants, which showed more rapid necrosis and earlier leaf drop than control plants. All drought-treated plants grew slower and had reduced stomatal conductance, rates of CO_2 assimilation and transpiration compared to well-watered controls. There were no differences in these measures between the control plants and RNAi lines (Figures S5 and S6).

Chlorophyll fluorescence measurements indicated that the MYB134-RNAi transgenics had a greater reduction in F_q'/F_m' than WT under drought, a trend which continued through to the recovery period (Figure 3a). MYB134-RNAi plants were clearly more susceptible to the effects of drought than WT, although the difference to WT was less pronounced than with overexpressors. To test if the PSII damage in the MYB134-RNAi plants correlated with ROS and lipid peroxidation as we observed previously, both H_2O_2 and MDA were quantified. Under drought stress, three out of the four independently transformed low-CT lines had higher H_2O_2 content than WT, whereas under well-watered conditions, only one line displayed significantly elevated H_2O_2 (Figure 3b). Similarly, the MDA content of the different lines did not differ under non-stressed conditions, but following drought stress, all four RNAi lines showed significantly higher MDA concentrations than WT (Figure 3c). Again, we observed a correlation between H_2O_2 and MDA content. These data suggest that damage to PSII in the MYB134-RNAi low-CT transgenics is the result of greater ROS accumulation and oxidative damage relative to WT.

3.3 | Leaf necrosis is inversely proportional to CT content in drought-stressed poplar plants

In addition to showing reduced physiological and metabolic stress indicators, the high-CT transgenics also showed lower rates of leaf necrosis than WT during drought stress (Figure 4). During drought, a distinct zone of brown tissue formed along the leaf lamina edge, beginning with the oldest leaves. The severity of necrosis was quantified by the number of new necrotic leaves appearing per week. This parameter was inversely proportional to CT concentration. High-CT transgenic poplar saplings were clearly delayed in the development of necrotic tissue during drought stress and thus developed fewer necrotic leaves during drought stress than WT plants (Figure 4c). In general, the high-CT transgenics took 17–27 days to show any necrosis, whereas WT plants had symptoms after only 10 days (Figure S7). By contrast, necrotic symptoms appeared at approximately the same rate in the MYB134-RNAi plants compared to controls. However, the severity of the necrosis was greater in the MYB134-RNAi lines, which showed more necrotic leaves per week of drought than WT (Figure 4d). We note that in MYB134-RNAi plants, the CT concentrations were only four- to five-fold reduced relative to WT, compared to a 50-fold increase in CT content in MYB overexpressors. This likely explains why the MYB134-RNAi transgenic experiments showed more subtle differences relative to the WT plants. Nevertheless, in both experiments, we observed less necrosis with greater CT content.

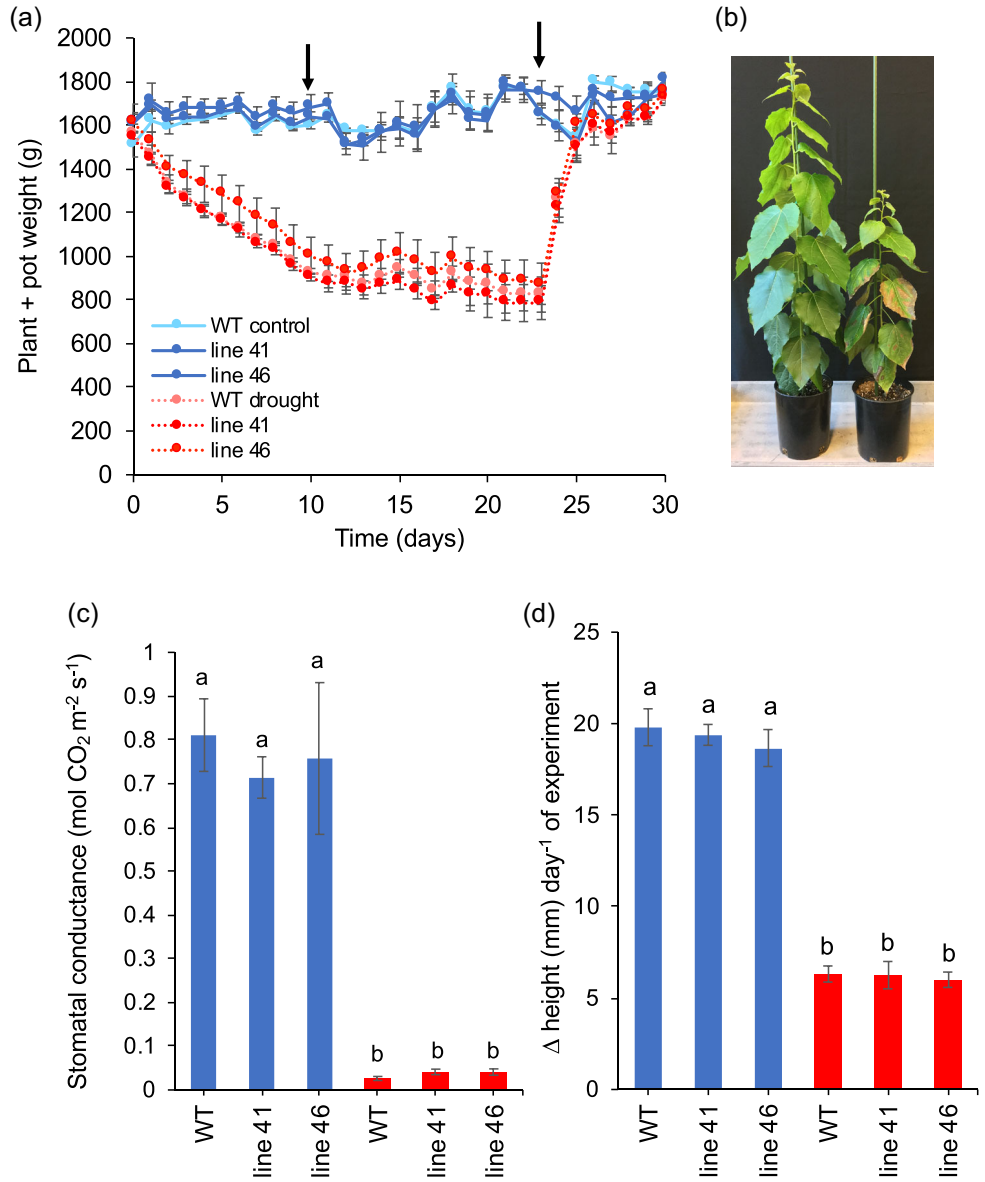

FIGURE 1 Time course of drought and impact on stomatal conductance and growth of wild-type (WT) and high-CT MYB134-overexpressing poplars (*P. tremula x tremuloides*). (a) Change in plant + pot weight during drought imposed as described in Materials and Methods (for MYB115-overexpressor data, see Figure S1). Onset of drought (Day 10) and recovery period (Day 24) are indicated with vertical arrows. (b) Representative image of well-watered and drought-treated WT plants. (c) Stomatal conductance at the end of drought (Day 23). (d) Plant growth rates for high-CT MYB134-overexpressor plants as a change in height between beginning and end of the drought. Letters indicate significant pairwise differences using Tukey's HSD test ($p < 0.05$). Red traces and bars indicate drought treatment, blue indicates well-watered. Lines 41 and 46 are independent high-CT MYB134-overexpressing transgenic lines. Data points are the means of four measurements for four replicate plants of each line. Error bars represent SE ($n = 4$). [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Impact of UV-B exposure on growth, photosynthesis and secondary compounds in MYB134-overexpressing and WT poplars

To determine if CTs can protect poplar leaves against oxidative conditions caused by a different abiotic stress, we exposed high-CT transgenic plants to UV-B radiation. We used custom-built sun simulator environmental chambers at the Helmholtz Zentrum, München, enabling close-to-natural spectral irradiation including UV-B. Due to the constraints of shipping plants, only a subset of transgenic lines was tested. We first confirmed the effects of UV-B exposure on WT plants. As predicted, after 2 weeks of UV-B exposure, MYB134 transcripts and CT levels increased dramatically (Figure 5). No induction of CTs was found in either of the high-CT transgenic lines, with their constitutively high CT levels (Figure 5). An increase in anthocyanin content in young leaves was observed following UV-B exposure, but there were no differences between transgenics or WT (Figure 5c). We observed a slight increase in

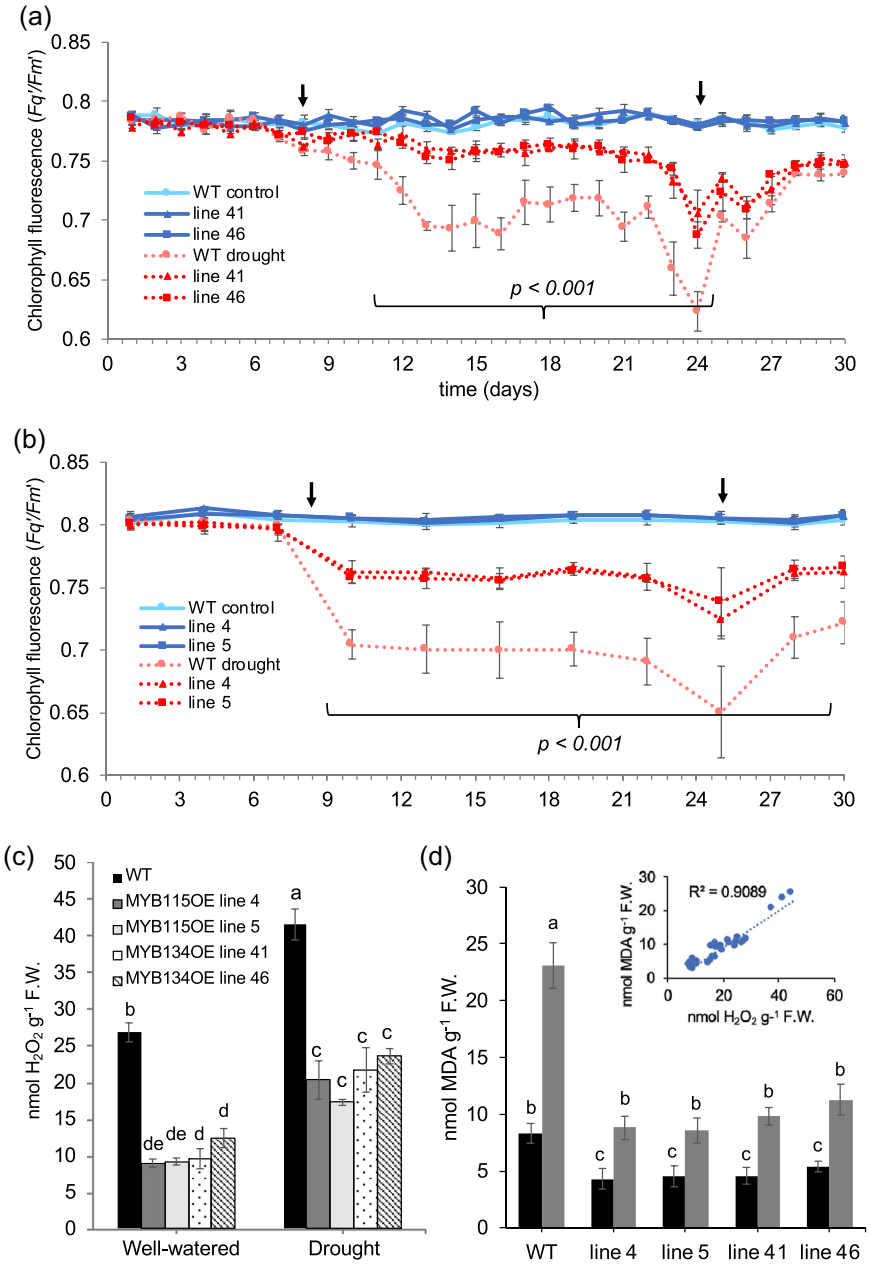

FIGURE 2 Impact of drought on chlorophyll fluorescence and H_2O_2 and malondialdehyde content in high-CT MYB-overexpressing transgenic and wild-type (WT) poplar plants. Chlorophyll fluorescence (F_q'/F_m') time course for (a) high-CT MYB134-overexpressing and (b) MYB115-overexpressing plants compared to WT controls. Red dashed lines indicated drought-stressed plants, solid blue lines indicate well-watered plants. Vertical arrows mark the beginning of drought (Day 8) and recovery period (Day 24 and Day 25 for panels a and b, respectively). Significant differences in fluorescence between each transgenic line and WT in drought-stressed plants are indicated by horizontal brackets (repeated measures ANOVA; $p < 0.001$). (c) H_2O_2 content in leaves of high-CT and WT saplings after drought. (d) MDA content in leaves before (black bars) and after (grey bars) drought stress for high-CT and WT plants. Lines 4 and 5 are MYB115-overexpressors, lines 41 and 46 are MYB134-overexpressors. Letters indicate significant pair-wise differences using Tukey's HSD test ($p < 0.05$). All data points are the means of four measurements for four replicate plants for each independent line. Error bars represent SE ($n = 4$). Inset shows a correlation of MDA levels with H_2O_2 content. [Color figure can be viewed at wileyonlinelibrary.com]

relative flavonol content in young and mature leaves for both plant types due to UV-B. Chlorophyll content was not impacted by UV-B exposure (Figure S8).

We also measured the emission of isoprene, a stress response in poplar affected by high temperature and strong light. Isoprene emission was not significantly affected by UV-B exposure (Figure 6). However, the high CT transgenics generally emitted less isoprene than WT. In the 2-week experimental period, we observed no effect of UV-B on growth, and no significant differences in plant growth as measured using height or stem diameter between WT or high-CT lines (Figure S9a,b). An increase in stem diameter appeared to be slightly lower in both line 41 and line 46 compared to WT, but this difference was not significant ($p < 0.1$ UV-B (−) and $p < 0.3$ UV-B (+)). There were also no differences in lamina length between transgenic lines or in response to UV-B (Figure S9c,d). Photosynthetic rate and WUE were generally not affected by UV-B, but transpiration rate and stomatal conductance decreased in UV-B-exposed plants compared to plants without UV-B (Figure S10). However, there were no differences between the transgenic lines and WT plants in these parameters.

3.5 | High CT content also protects poplar leaves against photosystem damage by UV-B radiation

Plants grown in the sun simulators irradiated with the UV-A, visible and near-infrared part of the solar spectrum but without the UV-B radiation showed stable chlorophyll fluorescence measurements for both light-adapted (F_q'/F_m') and dark-adapted (F_v/F_m) parameters

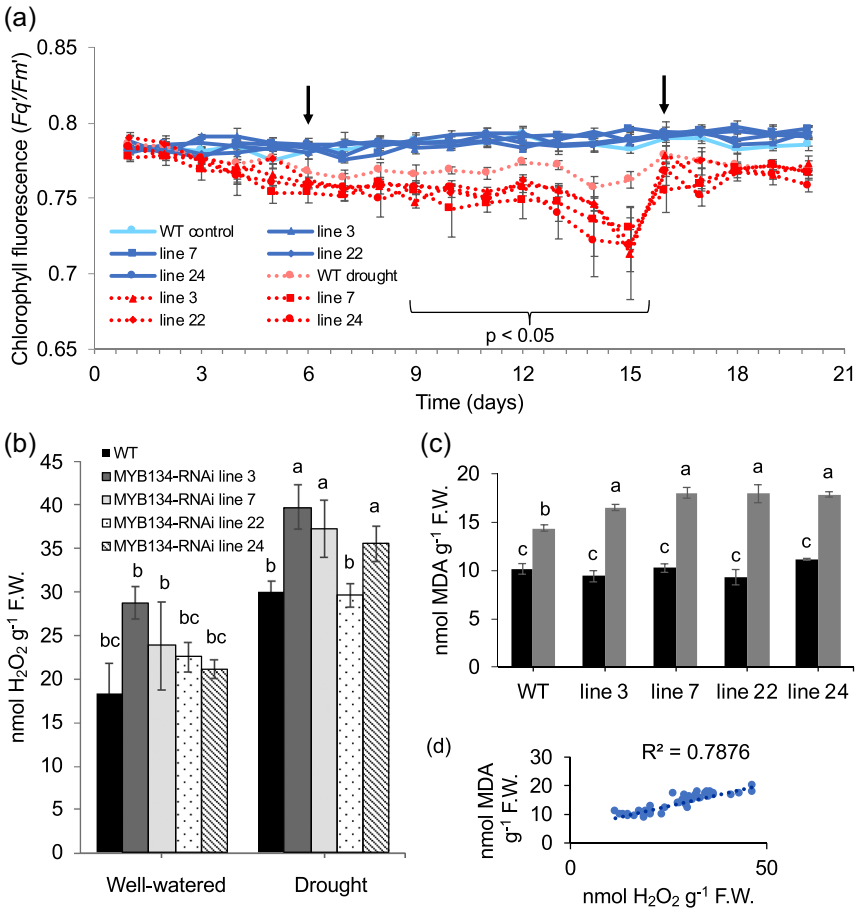

FIGURE 3 Impact of drought on chlorophyll fluorescence and H_2O_2 and malondialdehyde (MDA) content in low-CT MYB134-RNAi and wild-type (WT) control plants. (a) Chlorophyll fluorescence (F_q'/F_m') time course for MYB134-RNAi and WT plants during drought. Red dashed lines indicate drought-stressed plants and solid blue lines indicate well-watered plants. Vertical arrows indicate beginning of drought (Day 6) and recovery (Day 16). Significant differences in fluorescence between each transgenic line and WT in drought-stressed plants are indicated by horizontal brackets (repeated measures ANOVA; $p < 0.05$). (b) H_2O_2 levels for low-CT and WT plants with and without drought treatment. (c) MDA levels before (black bars) and after (grey bars) drought stress for low-CT and WT plants. Letters indicate significant pair-wise differences using Tukey's HSD test ($p < 0.05$). Data points are the means of measurements from four replicate plants for each independent transgenic line. Error bars represent SE ($n = 4$). (d) Correlation of MDA and H_2O_2 levels. [Color figure can be viewed at wileyonlinelibrary.com]

throughout the 2-week experimental period (Figure 7). However, WT plants grown with UV-B radiation showed a reduction in fluorescence for both parameters, falling from 0.78 at the beginning to around 0.7 by the end of the experiment. By contrast, no reduction in chlorophyll fluorescence was detected in high CT transgenics under the UV-B regime (Figure 7a). This indicates that the high-CT transgenics were better protected from the damaging effects of UV-B radiation than WT plants. The same effect was seen for both F_q'/F_m' and F_v/F_m: in both cases, only the WT showed a decrease in PSII fluorescence due to UV-B exposure (Figure 7b). Therefore, for both light- and dark-adapted fluorescence parameters, our data indicated that high CT plants retained normal PSII function during UV-B stress.

Similar to what we observed following drought stress, the H_2O_2 content in the high-CT transgenics was lower compared to WT. Although the UV-B treatment induced elevated H_2O_2 accumulation in the high-CT transgenics, the increase was greater in WT plants (Figure 7c). Likewise, leaves of WT plants had significantly higher levels of MDA compared to the high-CT transgenics after UV-B exposure (Figure 7d). Again, in both transgenic and WT plants, MDA levels increased following UV-B exposure, but less in the transgenic lines. In general, the increase in H_2O_2 and MDA under UV-B radiation matched the reduction in PSII function observed in WT plants (Figure 7).

4 | DISCUSSION

4.1 | CTs are cellular antioxidants in poplar trees

Condensed tannins have inherent antioxidant capacity (Hagerman et al., 1998), but it is not known if they contribute to oxidative stress resistance in vivo. Here, we used transgenic poplars to demonstrate that CTs can reduce the negative oxidative effects of two different abiotic stresses. In both drought and UV-B experiments, high-CT

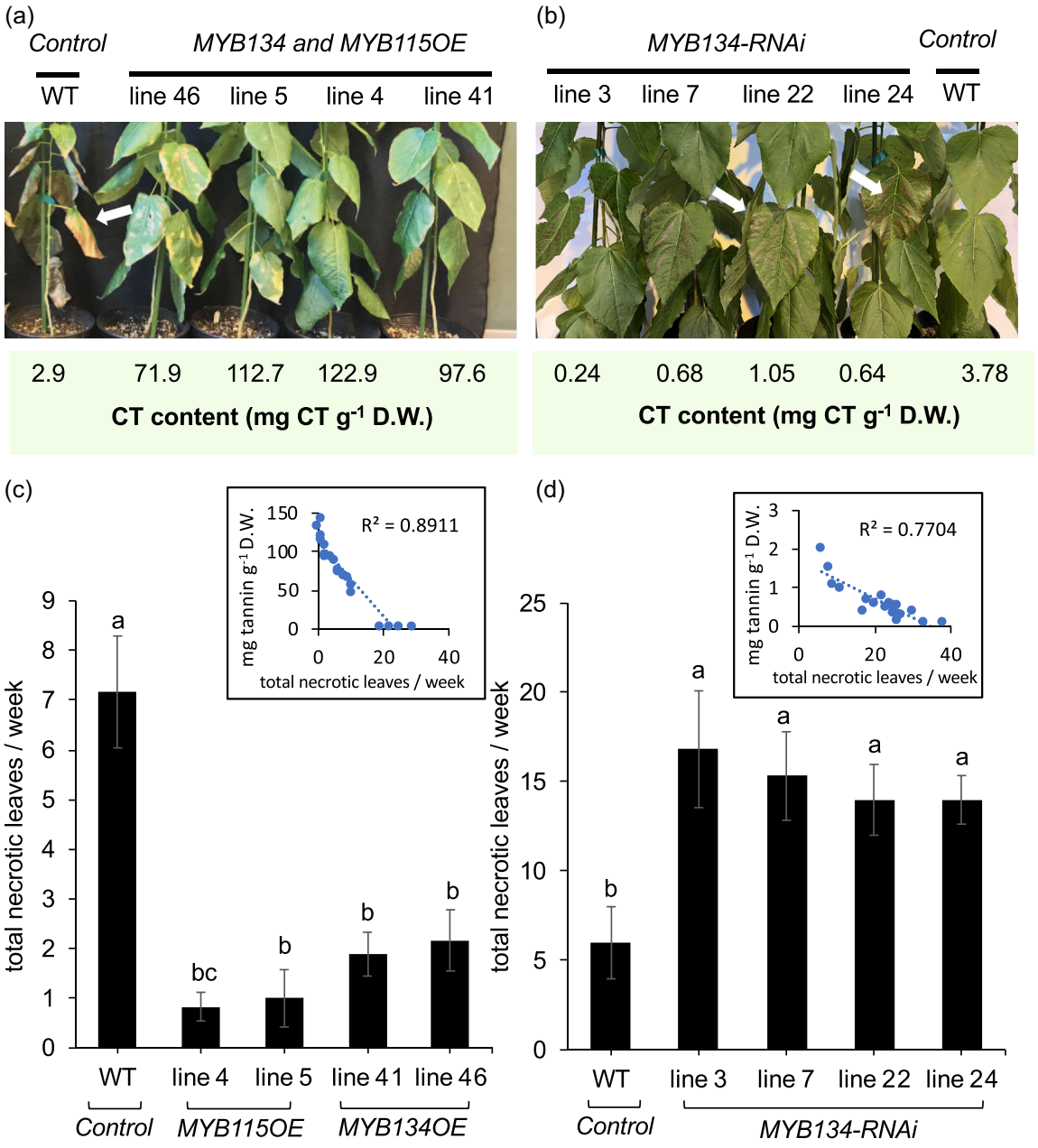

FIGURE 4 Development of leaf necrosis in drought-stressed poplar. Top panels show representative images of necrosis on leaves of high-CT MYB-overexpressor (a) and RNAi-suppressed low-CT transgenics (b) compared to wild-type (WT) plants after drought. CT concentrations are shown below the image for each plant. White arrows emphasize the necrotic tissue due to drought stress. (c) Rate of necrosis development in WT and high CT MYB-overexpressing poplar expressed as new necrotic leaves/week over the course of the entire experiment. (d) Necrosis development in low-CT transgenic MYB134-RNAi plants compared to WT plants. Letters indicate significant pair-wise differences using Tukey's HSD test ($p < 0.05$). Error bars represent SE ($n = 4$) for each transgenic line. Insets show correlations of CT content with the rate of necrosis development. [Color figure can be viewed at wileyonlinelibrary.com]

poplar had less H_2O_2 and lower MDA content and demonstrated less PSII damage compared to controls. Plants with suppressed CT content showed the opposite pattern for these parameters after drought. Finally, drought stress-generated leaf necrosis was inversely correlated with CT content.

These results shed new light on the biological functions of CTs. Earlier work with high-CT poplar plants had focused on biotic stresses (Boeckler et al., 2014; Ullah et al., 2017), but the impact of high CT content on abiotic stress resistance on whole plants had not been tested. Previous observations had shown that UV-B radiation induces CTs synthesis and that high CT plants performed somewhat better than control plants under UV-B stress (Mellway & Constabel, 2009; Mellway, Tran, et al., 2009). The current experiments demonstrate a clear protective effect of CTs against drought and UV-B. Together with our previous work showing greater tolerance towards the ROS-producing herbicide methyl viologen

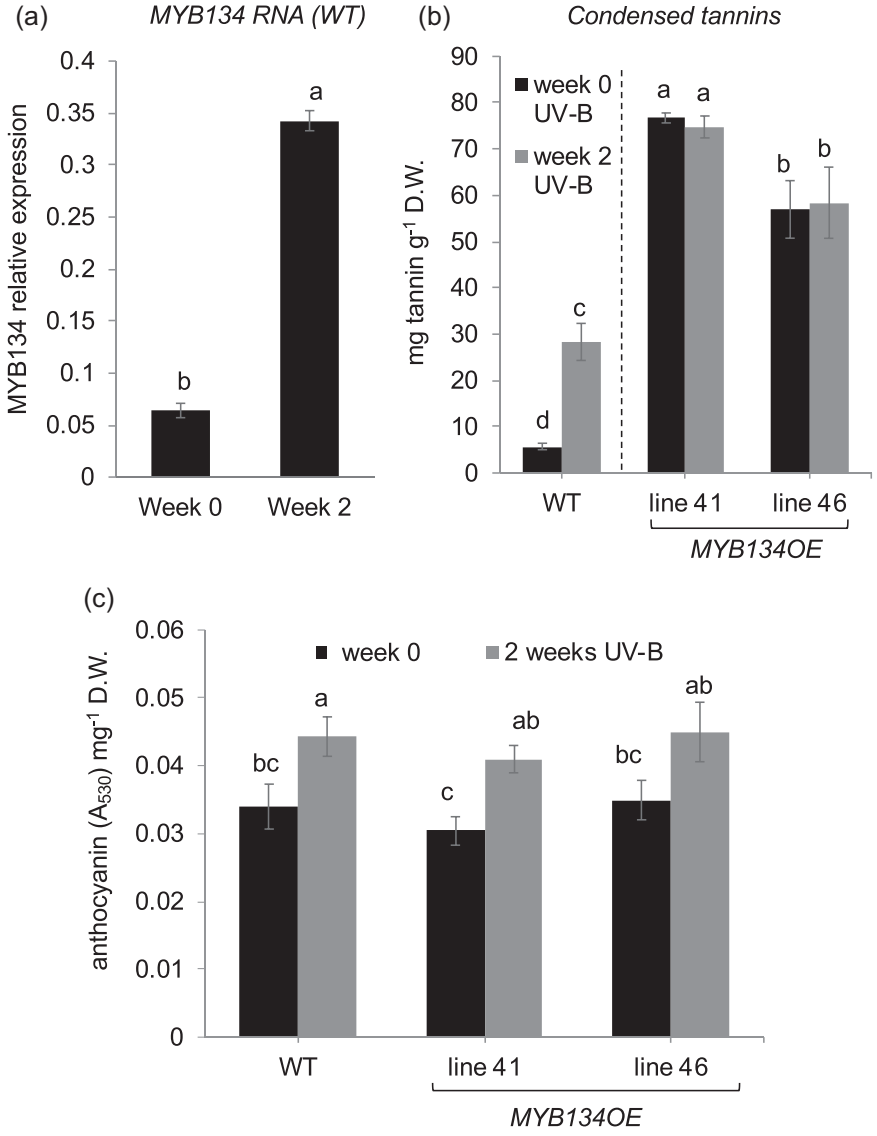

FIGURE 5 Induction of MYB134 transcripts, condensed tannins (CT) and anthocyanin in wild-type (WT) and high-CT MYB-overexpressing poplar after a 2-week UV-B exposure. (a) MYB134 relative transcript abundance in WT poplar assayed by quantitative polymerase chain reaction, normalized with elongation factor 1β and ubiquitin transcripts. (b) Concentration of condensed tannins in high-CT MYB134-overexpressor lines and WT plants as quantified by butanol-HCl assays. (c) Anthocyanin concentration, assayed spectrophotometrically as outlined in the Materials and Methods. Letters indicate significant pair-wise differences using Tukey's HSD test ($p < .05$). Data points represent the means of six biological replicates (individual plants). Error bars are SE ($n = 6$)

(Gourlay & Constabel, 2019; Gourlay et al., 2020), these data demonstrate that CTs can protect leaves against ROS-generating abiotic stresses. Future experiments will test if these short-term effects also translate into enhanced productivity and growth potential.

4.2 | Chlorophyll fluorescence demonstrates protective effects of CTs and detects distinct patterns of photosystem damage after drought and UV-B

Our experiments showed a negative impact on PSII fluorescence and demonstrated that CTs mitigate these effects against both abiotic stresses tested. Nevertheless, we detected differences in the types of photosystem damage. Both drought and UV-B stress led to decreased F_q'/F_m'. This chlorophyll fluorescence parameter estimates the proportion of absorbed light that is actually used for PSII photochemistry, that is, the PSII operating efficiency (Murchie & Lawson, 2013). UV-B, but not drought, additionally reduced F_v/F_m. This parameter is a measure of the maximum quantum yield of PSII; photoinhibition or damage to the photosystems will be manifested as a reduction in F_v/F_m. The impact of UV-B on F_v/F_m, therefore, suggests greater or more long-lasting damage to the photosynthetic apparatus compared to drought. This is consistent with what is known about how both abiotic stresses impact plant function. Under drought, the damage is caused by ROS in photosynthetically active leaves only indirectly as a consequence of cellular dehydration and stomatal closure (Cramer et al., 2011). Dehydration reduces membrane stability, and electrons are diverted to oxygen from the thylakoid electron transport chain and generate ROS in close proximity to the photosystems (Smirnoff, 1993). Likewise, the reduced internal CO_2 concentrations during stomatal closure cause a build-up of excitation energy in photosynthetic electron transport, which can also generate superoxide (Cruz de Carvalho, 2008). These ROS reduce PSII efficiency. By contrast, UV-B radiation can directly damage proteins associated with PSII, including the oxygen-evolving complex and quinone acceptor complexes. This leads to photosystem inactivation as well as ROS generation (Asada, 2006; Foyer et al., 1994; Kataria et al., 2014). Additional ROS are generated by

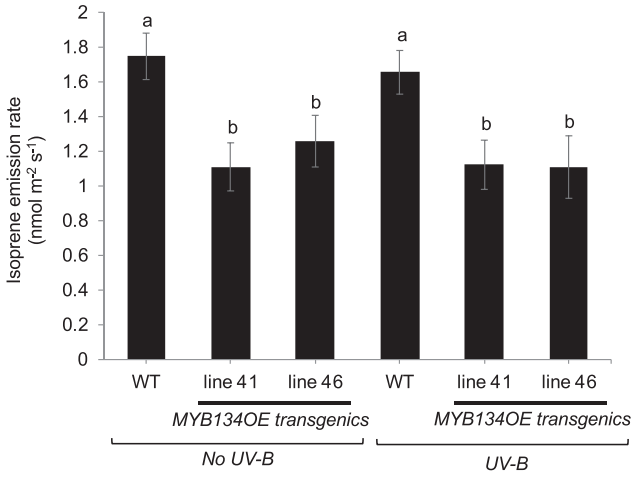

FIGURE 6 Isoprene emission rates in high-CT MYB134-overexpressor and wild-type (WT) poplar with and without UV-B exposure. After 2 weeks of UV-B exposure, isoprene emission rates were measured as described in Materials and Methods. Letters indicate significant pair-wise differences using Tukey's HSD test ($p < 0.05$). Data points are the means of six replicate plants for each independent transgenic line. Error bars represent SE ($n = 6$).

UV-B-damaged photosystems from leakage of electrons during photosynthetic electron transport (Takahashi & Badger, 2011). Ultimately, this damage is reflected in a decreased quantum yield of PSII (F_v/F_m). High CT concentrations protected leaves against this damage, however.

The lack of change in the F_v/F_m parameter in drought experiments suggested that PSII reactions centres were not inactivated or degraded by the short drought stress we applied here, although the measure of PSII efficiency (F_q'/F_m') was reduced. We note that the hybrid used in our work, *P. tremula* × *tremuloides*, is considered a moderately drought-tolerant poplar (Dickmann, 2001). Other work with the drought-sensitive *Populus* × *euramericana* hybrid 'Neva' found that both parameters were reduced by drought (Liang et al., 2019). Detecting the impacts of drought on PSII using dark-adapted parameters (F_v/F_m) might be possible if one imposed more extreme stress, as has been shown in other species (Kalaji et al., 2017; Wada et al., 2019). The effect of UV-B exposure on both F_v/F_m and F_q'/F_m' parameters was previously reported by Davey et al. (2012), who showed that exposing Arabidopsis to UV-B leads to reductions in both F_v/F_m and F_q'/F_m'. In tobacco, a negative effect of UV-B on F_v/F_m was also seen (Czégény, Le Martret, et al., 2016) Other studies, however, failed to show an impact on this parameter, for example in *P. cathayana* or grapevine (Martinez-Lüscher et al., 2013; X. Xu et al., 2010). Abiotic stress adaptations clearly vary widely among species.

4.3 | Potential mechanisms of condensed tannins for removing ROS

The mechanism(s) by which CTs can interact with or remove ROS still need to be elucidated. Like most flavonoids, the CTs are generally localized to the vacuole in living cells (Abeynayake et al., 2011; Gutmann & Feucht, 1991). By contrast, ROS are produced mostly in the chloroplast, mitochondria and peroxisomes (Dat et al., 2000). The primary site of H_2O_2 production in leaf mesophyll cells during stress is the chloroplast (Behnke et al., 2010), as light energy exceeds the capacity for carbon assimilation or thylakoid membranes are damaged. The superoxide generated via the Mehler reaction is rapidly dismutated to H_2O_2 by plastidic SODs (Apel & Hirt, 2004). H_2O_2 is a relatively stable ROS; if not removed by catalases it can diffuse throughout the cell (Mittler, 2017). Furthermore, Agati and others have proposed that H_2O_2 can efficiently cross the tonoplast into the vacuole via aquaporins (Agati et al., 2012; Bienert et al., 2007). In mesophyll cells, the cytoplasm consists of a thin layer surrounding the large central vacuole, and H_2O_2 production in chloroplasts thus occurs in close proximity to the tonoplast and vacuole. Within the vacuolar compartment, the degradation of H_2O_2 involving APX (ascorbate peroxidase)-mediated oxidation of flavonols has been proposed, based on the high affinity of APX for flavonoids (Agati et al., 2012; Yamasaki et al., 1997). Whether such an APX mechanism could apply to the CTs as well still needs to be tested. CTs may also function directly as antioxidants, via single-electron transfer or H-atom transfer (Quideau et al., 2011). Independent of the mechanism, our data demonstrate that the presence of CTs is associated with reduced H_2O_2 content in leaves.

Although we only measured H_2O_2 here, previously we showed that superoxide accumulation parallels the increase in H_2O_2 (Gourlay & Constabel, 2019). Both of these ROS are produced in chloroplasts, which are found at high density in the palisade cells. Early histochemical work by Kao et al. (2002) in *P. tremuloides* localized the CTs to the palisade cells and lower epidermis. In MYB134 overexpressing poplar transgenics, CTs were likewise stained in the upper portion of the palisade layer, as well as the upper epidermis (Mellway, Tran, et al., 2009). Therefore, CTs are found within or near cells most active in photosynthesis, and in close proximity to ROS-producing areas of the leaf. The spatial correlation of ROS with the CTs thus supports our antioxidant hypothesis and is summarized in our functional model (Figure 8). The epidermal localization is also relevant for the direct absorption of UV-B radiation by CTs (see below).

4.4 | Does CT act as a UV-B filter?

Despite the clear effect of UV-B radiation on chlorophyll fluorescence in WT poplars, neither fluorescence parameter was impacted in the high-CT transgenics. Thus, both F_q'/F_m' and F_v/F_m time course profiles were indistinguishable from those for WT plants under non-UV-B conditions (Figure 7). The lack of a negative impact of UV-B on chlorophyll fluorescence parameters in the high-CT transgenics was surprising and unlike the effects of drought (Figure 3). These data suggest that in high CT transgenics, PSII was extensively protected from the damaging effects of UV-B damage. This difference with drought stress could again reflect the distinct mechanisms by which the two stresses generate ROS. We speculate that CTs may additionally help to protect against UV-B stress directly by acting as a UV-B screen, and absorbing UV wavelengths and preventing them from penetrating into the leaf.

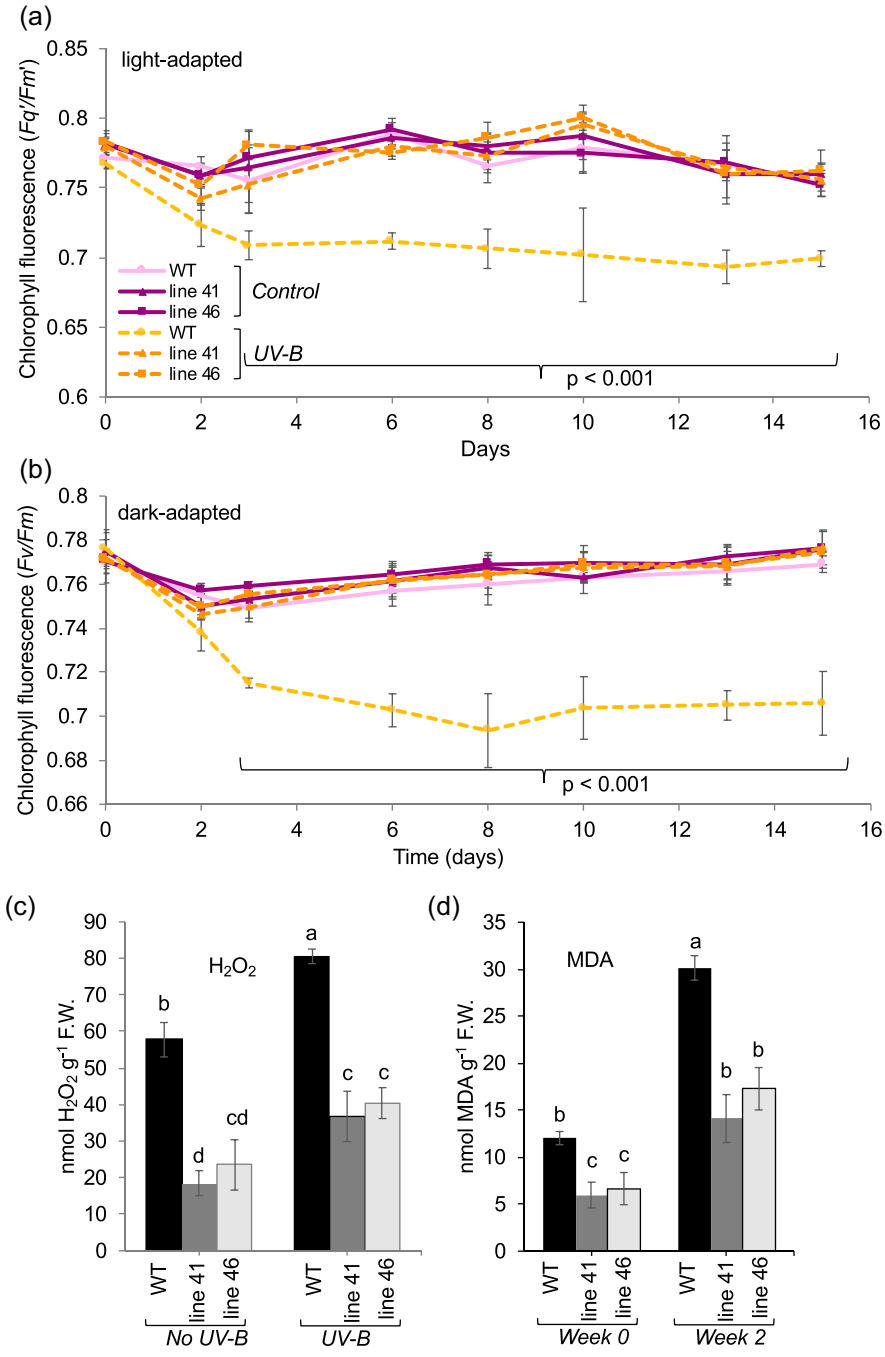

FIGURE 7 Impact of UV-B on chlorophyll fluorescence, malondialdehyde (MDA) and H_2O_2 content in high-CT MYB-overexpressing transgenic and wild-type (WT) poplars. Plants were exposed to UV-B radiation for 15 days as outlined in Materials and Methods. Top panels show the light- and dark-adapted chlorophyll fluorescence during UV-B exposure in high-CT transgenic and WT poplar using F_q'/F_m' (a) or F_v/F_m (b). Significant differences to WT of UV-B exposed plants in chlorophyll fluorescence are indicated with horizontal brackets (repeated measures ANOVA; $p < 0.001$). Lower panels show H_2O_2 content at the end of the experiment in no-UV-B and UV-B-exposed plants (c) and MDA content before and after UV-B-stress (d). Letters indicate significant pair-wise differences using Tukey's HSD test ($p < 0.05$). Lines 41 and 46 are independent MYB134-overexpressing transgenic lines. Data points are the means of six biological replicates. Error bars represent SE ($n = 6$). [Color figure can be viewed at wileyonlinelibrary.com]

The localization of CTs in MYB134-overexpressors to the upper epidermis and palisade cells (Mellway, Tran, et al., 2009) supports this hypothesis. By contrast, leaves of greenhouse-grown WT saplings contain CTs mostly in the lower epidermis. Most photosynthesis and chlorophyll fluorescence activity occur in the palisade cells located directly below the upper epidermis (Kalaji et al., 2017). In the high-CT plants, CTs in the palisade cells could thus shield excess UV-B and prevent photosystem damage (Figure 8). To our knowledge, CTs have never been examined in this context. In solution, flavan-3-ols and CTs have absorption maxima near 280 nm but do absorb in the 280–315 nm range as well (Huvaere & Skibsted, 2015; Laghi et al., 2010). Furthermore, the role of phenolic acids and flavonoids in direct UV-shielding has been documented (Burchard et al., 2000), supported by localization of these compounds to epidermal cells (Hutzler et al., 1998; Kaling et al., 2015).

We observed that even the high-CT leaves manifested an induced accumulation of foliar anthocyanins and flavonols after the UV-B treatment (Figure 5, Figure S8). Therefore, at least some cells experienced sufficient UV-B radiation to induce the synthesis of these compounds. In addition, MDA and H_2O_2 levels in the high-CT transgenics increased in response to UV-B radiation, indicating that at least some cellular damage and lipid peroxidation had occurred (Figure 6). To better understand how UV-B interacts with CTs, more spatially explicit experimental strategies to determine where ROS is

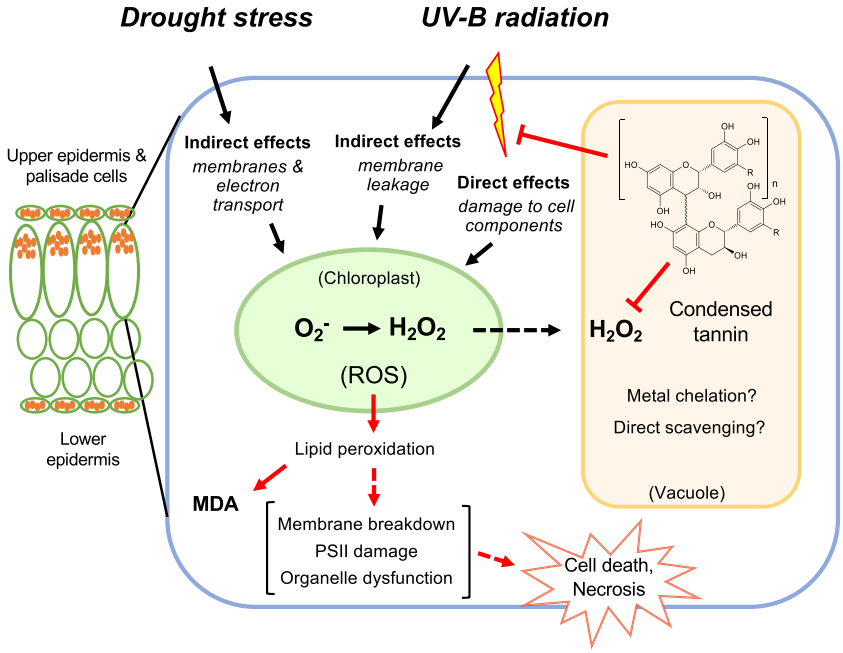

FIGURE 8 Model of direct and indirect effects of stress on leaf palisade cells and the proposed interactions of reactive oxygen species (ROS) with condensed tannins (CTs). CTs (brown colouration) are located in the palisade and epidermal cells within vacuoles and in close proximity to chloroplasts, which allows them to participate in the removal of H_2O_2. ROS are generated in chloroplasts via both direct and indirect effects of stress. Superoxide (O_2^-) is formed in chloroplasts when photosynthetic membranes are damaged or the photosynthetic electron transport chain in Photosystem I is over-reduced. H_2O_2 is formed by dismutation of O_2^-. It is more stable and can diffuse throughout the cell and be transported into the vacuole where it interacts with CTs. These may act directly as antioxidants, or chelate Fe to prevent its interaction with H_2O_2 and the formation of hydroxyl radicals. Additional protection against UV-B is hypothesized to occur via direct absorption of UV irradiation by CTs. Excessive ROS accumulation in chloroplasts and cells leads to lipid peroxidation and membrane breakdown, and ultimately cell death. Malondialdehyde (MDA) is a product of lipid peroxidation and a marker of oxidative cell damage. [Color figure can be viewed at wileyonlinelibrary.com]

produced and where CTs accumulate will be needed. For example, H_2O_2 could be fluorescently imaged in live leaves (Behnke et al., 2010), and flavonoids and phenolics imaged using specific dyes and confocal laser microscopy (Hutzler et al., 1998; Kaling et al., 2015). Applying such techniques to CTs in poplar will help to resolve these discrepancies.

Additional clues may come from the analysis of other antioxidant metabolites. We measured the emission of isoprene, a volatile that has been extensively studied for its role in abiotic stress responses. It is emitted in particular during heat stress and has been hypothesized to help stabilize membranes and prevent lipid denaturation following oxidative stress (Behnke et al., 2007) or to regulate the production of ROS via protein nitrosylation (Vanzo et al., 2016). In our experiments, UV-B did not increase isoprene emission in poplar, similar to previous work (Kaling et al., 2015). However, overall lower rates of isoprene emission were observed in the high-CT transgenic poplar (Figure 6). This could be due to a compensatory response, or metabolic trade-offs. However, previous work showed that in transgenic poplars with no isoprene emission, the phenylpropanoid pathway was down-regulated (Behnke et al., 2010; Kaling et al., 2015; Monson et al., 2020). Metabolic cross-talk between the isoprenoid and flavonoid pathways based on the enzyme chalcone isomerase has been demonstrated in tomato (Kang et al., 2014).

In conclusion, we provide direct evidence that CTs can contribute to resistance against abiotic stress. Despite fundamental differences in how drought and UV-B stress impact plant cells, the production of ROS and oxidative stress is common to both stresses. CTs have excellent antioxidant activity in vitro, and our data provide the first evidence that they have this capacity in an in planta context as well. Given that CTs comprise a large proportion of fixed carbon in forest ecosystems, these physiological and ecological roles could have large-scale consequences.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Geraldine Gourlay, Barbara Hawkins, Jörg-Peter Schnitzler and C. Peter Constabel designed and planned the research. Geraldine Gourlay performed experiments and analysed the data. Andreas Albert helped with designing, executing and interpreting experiments. Geraldine Gourlay, Barbara Hawkins, Jörg-Peter Schnitzler and C. Peter Constabel wrote and edited the manuscript. C. Peter Constabel agrees to serve as the author responsible for contact and communication.

DATA AVAILABILITY STATEMENT

The data that support this study are available from the corresponding author upon request.

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